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**Molecular Detection of Host-Specific Fecal Bacteria in Three Major Watersheds of
Piedmont Georgia**

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Department of Biological and Environmental Sciences in fulfillment for the degree of Master of
Science Georgia College and State University

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Abstract

Fecal pollution is a major concern in creeks and rivers. The aim of this study was to enumerate fecal coliform bacteria and identify the source of contamination using Microbial Source Tracking at three different creeks (Mountain Oak, Long Cane, and Ulcohatchee) in the Piedmont region of Georgia. Data collected in this research will be used to formulate watershed rehabilitation plans to decrease the transfer of fecal indicator bacteria (FIB) into these creeks. Samples were collected at several sites along the creeks (4 sites at Mountain Oak, 10 sites at Long Cane, and 5 sites at Ulcohatchee). The site locations are near artificial land cover such as roads, bridges, or houses. IDEXX analysis was performed to quantify fecal coliform (FC) bacterial levels and qPCR using human and animal specific Bacteroidales markers was used for Microbial Source Tracking (MST). FC pollution was highest in the warmer months: June through October in the Mountain Oak creek, April through September in the Long Cane creek, and August in the Ulcohatchee creek. Some measurements exceeded the USEPA guidelines of 200 MPN/100ml for FC bacteria in recreational waters. The highest value of 1986.3 MPN/100ml was detected at site 1 at the Mountain Oak Creek in June. In the Long Cane creek, the maximum value of 933.3 MPN/100 ml was recorded at site 2 during September 2019. The maximum value at the Ulcohatchee creek was 1299.7 MPN/100ml which was recorded in August 2018 at site 1. Microbial source tracking analysis showed that horse was the major source of fecal indicator bacteria (FIB) in Mountain Oak creek and was detected mostly at sites 1 and 4. Cattle and deer were the major contributors in the Ulcohatchee creek. The cattle marker was detected on 7 different occasions at this creek and was detected on two separate accounts at sites 1 and 2. These two sites also had the highest overall average FC levels out of the five sites at this creek. In the Long Cane creek, beaver was the main source of FIB because it was detected at every site

at least once during the three source tracking events. Cattle and the deer markers were most common in sites 5 and 6 of this watershed.

Introduction

Fecal coliform (FC) bacteria are used as indicators for testing water quality in wetlands such as streams, creeks, and rivers. These bacteria are found in the gut of humans as well as herbivorous and carnivorous animals such as birds, ruminants, and canines (Lama and Bachoon, 2018). They are spread in the environment via point and non-point sources such as sewage, septic tanks, factories, wastewater treatment plants, fecal matter from runoff, wildlife, and agriculture. In Georgia, creeks and streams that empty into larger water bodies can become contaminated from non-point sources of pollution. Human recreational activities such as swimming and fishing can also increase the rate of transport of bacteria into surface water. Increased runoff from impervious cover such as roads, sidewalks, parking lots, and bridges can travel to waterways (Paule-Mercado et al. 2016). Farming practices and land that supports cattle, horses, hogs, and chicken are also another non-point source of bacteria. Wildlife such as white-tailed deer, coyote, beaver, and wild boar are common in the state of Georgia. Finally, household pets such as dogs and cats can often distribute FIB into water. Most surface waters including lakes, rivers, streams, and marine environments are used for drinking water supply and recreation purposes. Ingestion or exposure to contaminated water poses a serious public health risk. FC bacteria alone are not considered harmful but high levels in the environment indicate the presence of pathogenic bacteria, viruses, and protozoans. These pathogens include *Giardia*, *Shigella*, *Salmonella*, and *E. coli*. Thus, high loads of indicator bacteria such as FC or *E. coli* in recreational water are considered a risk factor for developing gastrointestinal illnesses which can potentially lead to death (Wang et al. 2002).

To assess the impairment of water and risk to human health, the US EPA has developed specific criteria for monitoring environmental water quality in the United States. The EPA focuses on FIB such as *Escherichia coli* or fecal coliform to test the level of contamination in recreational waters (US EPA, 2012). In order for a FIB to be an effective indicator, it must (i) not survive for prolonged periods in the environment (ii) it must be pathogenic to humans (iii) it has to be associated with the intestinal tract of warm-blooded animals. The US EPA recommends *E. coli* and fecal coliforms for fresh and marine waters while enterococci are more effectively used for testing marine waters. The fecal coliform Single Sample Maximum standard for safe freshwater recreational use of 200cfu/100ml was established in the United States for recreation (US EPA, 1976). It is very important to identify the source or sources of fecal contamination before effective steps can be taken to mitigate the problem and lower the level of fecal bacteria in the water.

To determine the source of fecal contamination, recently scientists have used Microbial Source Tracking to target host specific *Bacteroides* (Bachoon et al. 2010, Hernandez et al. 2013, Zang et al. 2019). Members of this genus are classified in the Phylum Bacteroidetes, which are described as Gram negative rod shaped with a variety of physiological types, from being completely anaerobic *Bacteroides* to being aerobic Flavobacteria (Thomas et al. 2011). These bacteria comprise 25% of fecal mass and Bacteroidale-based methodologies are designed to target host-specific 16 rRNA gene present in feces from different host animals. *Bacteroides* are commonly found in the host microbiota and some exhibit a greater affinity for their host than others. For example, the Hf183 human marker demonstrates a high level of host-specificity and

sensitivity to humans (Ahmed et al. 2016, Hernandez et al. 2013, Staley et al. 2012). Being obligate anaerobes, *Bacteroides* have limited survivability in the environment and they lose culturability after a few days post-deposition. However, it has been demonstrated that PCR amplifiable nucleic acid sequences in these markers can persist in the environment for much longer (Okabe and Shimazu 2007). Decay rates of these markers may vary with increasing temperature, sunlight, and salinity. Recently, specific *Bacteroides*-based MST assays have been developed for a wide range of species such as human, cow, canine, deer, horse, chicken and other birds, pig, and beaver. In Georgia, MST research in small streams has been effectively demonstrated. Bachoon et al. (2009), showed that the major source of contamination in the Lake Oconee watershed comes from cattle and humans. Source tracking was performed using polymerase chain reaction with *Bifidobacterium adolescentis* and *Bacteroides* (BoBac) as markers for human and bovine fecal pollution. This study did not assess other possible sources such as wildlife and poultry. Other research studies have demonstrated the use of *Bacteroides*-specific microbial trackers as predicting variables for the presence of fecal contamination. Bradshaw et al. (2016) demonstrated that high FIB concentrations may not indicate presence of specific pathogens in stream water. Additionally, the results of the study support that the presence of FIB does not directly suggest presence of certain *Bacteroides* markers. However, Bradshaw et al. (2016) did show positive correlation between the CowM3 and Rum-2-Bac markers and the presence of pathogenic Shiga toxin producing *E. coli*. MST assays targeting *Bacteroides* may provide better indication of more recent fecal contamination than fecal coliform or *E. coli* because they do not survive for extended periods in the environment. They are also more abundant in the intestinal tract of warm-blooded animals (Fiksdal et al. 1985).

River Basin Background

This research examines water quality of three watersheds located in the two major Piedmont river basins (middle Chattahoochee and the upper Flint). The Southern Piedmont is in Eastern United states and covers 16.5 million hectares, extending from Alabama to Virginia. This region is characterized by rolling topography and ample precipitation. The lower Piedmont region of Georgia contains the Chattahoochee, Flint, Altamaha, Savannah, and small portions of the Ogeechee river. The middle Chattahoochee river basin covers 915 square miles and serves as a primary source of treated wastewater for 3.5 million people in the Atlanta Metro District (Regional Commission [ARC], 2010). This river basin is part of the piedmont province and covers six counties (Cobb, Clayton, Coweta, Douglas, Fulton, and Paulding) within the Metro Water District portion. The topography of this region is highly variable with rolling hills and isolated mountains. The Chattahoochee flows to the Gulf of Mexico after joining the Flint river in the Southern part of Georgia. Many aquatic and semi-aquatic species of native flora and fauna exist in this watershed. Some of these species which include birds, fish, and invertebrates are protected. They are found in the district counties along the streams and creeks as well as the river margins. The Georgia Environmental Protection Division (EPD) determines the impairment of waterbodies through the collection of data and comparing them against the water criteria. Georgia 305(b)/303(d) integrated report of the Clean Water Act requires the state to assess its water quality biannually as well as develop Total Maximum Daily Load (TMDL) for all water that are not supporting their designated uses. In 2014, approximately 255 miles of stream or 72% of assessed streams were labeled as impaired for violation of the water quality standards in the Metro District of the Middle Chattahoochee basin (GA EPD, 2014). Out of the 255 miles of streams, approximately 183 miles or 72% were labeled as impaired for violation of fecal

coliform criteria resulting from nonpoint source pollution. Fecal coliforms are found in both developed and underdeveloped streams/watersheds. These bacteria can increase in water due to high population density and anthropogenic activities as well as decreased stormwater filtration and treatment.

The upper Flint river basin is in the southern part of the Metro Water District and lies entirely in the Piedmont province. The basin area is characterized by rolling topography and isolated mountains and a gradual decrease in elevation from 1000 ft in the Northeast to 600 ft in the Southwest. The river originates in Atlanta and drains through Fayette, Clayton, Coweta, Douglas, and Henry counties eventually draining into the Gulf of Mexico at Apalachicola Bay. It contains approximately 240 miles of water in which 17% is designated for drinking, 18% for drinking and fishing, and 64% assigned for fishing. The groundwater aquifers are in crystalline rocks which are overlain by rock debris that is thickest in the valleys. These deposits do not provide a sufficient yield of water other than in low density areas thus surface water is the main source of drinking water for the District. There are several protected aquatic/semi-aquatic species found in the upper Flint river basin. They are highly impacted by increasing land use and impervious cover in the northern metropolitan areas. The rest of the upper Flint basin is characterized predominantly by underdeveloped land (24% agriculture, 40% forested, and 8% wetlands) as well as low density residential regions. Impervious cover does have a direct link to changes in hydrologic conditions of watersheds which affects stream stability, water quality, and biotic community integrity. Impervious levels of 10% or greater, have a detrimental impact on the biotic ecosystem and at levels greater than 25%, the streams cannot support viable communities (Schueler et al. 2001). In 2014, the GA EPD assessed the water quality of a total of 240 stream miles in the Metro Water District of the Upper Flint basin from which 124 miles, or

52%, did not meet the states standards on the 303(d) list. Out of the 124 miles, 104 miles, or 43%, did not meet the state standard for fecal coliform bacteria.

The Mountain Oak and Long Cane creeks are in the western Piedmont region and are part of the larger upper middle Chattahoochee watershed system. In recent years, increased urbanization has impacted the quality of the streams in the Southeast region of the U.S. Additionally, fecal coliform testing in the western Piedmont has been conducted to evaluate the impact of urbanization and increased impervious surface area on stream hydrology (Schoonover et. al, 2005) (Gregory and Frick, 2000). Schoonover reported a strong correlation between urbanization and increased fecal coliform and nutrient concentration in 16 watersheds that are connected to the Chattahoochee river basin. The FC concentration was the highest in urban and pasture areas in comparison to managed and unmanaged forested areas. Approximately 86% of the Mountain Oak creek is classified as agricultural land use, 0.01% commercial, 4.56% conservation, 0.77% exempt, 3.84% residential, 0.05% utility, 2.43% transportation, and 2.15% vacant/undeveloped. This creek travels through forested areas and occasionally intersects roads and bridges and nearby houses. The Long cane creek contains segments which are listed for fecal coliform and biota (fish) impairment on Georgia Environmental Protection Division's 2014. Within this watershed, 16.41% is classified as agricultural land use, 7.45% commercial, 29.63% conservation, 13.98% exempt, 6.38% industrial, 18.38% residential, 0.02% utility, 7.46% transportation, and 0.29% no classification. Increased anthropogenic activity in this region can impact the water quality of these streams. Previous research correlates increased fecal coliform counts in relation with increasing artificial land cover in the western Piedmont (Schoonover and Lockaby 2006). An increase in urban development means a higher frequency of water contamination through fecal runoff from livestock farming, pets, and human sewage. Runoff

from storm events can carry sewage contamination in urban areas (Olds et al. 2018). However, wildlife such as White-Tailed Deer, coyote, and beaver cannot be discounted as possible sources (Guber et al. 2015).

The Ulcohatchee creek is part of the upper Flint River watershed system that lies in Crawford county, Georgia. This creek was placed on Section 303(d) list by the GA EPD for violating the state standards for fecal coliform. The GA EPDs 2003 Total Maximum Daily Load Evaluation for twenty-eight stream segments in the Flint River basin called for a 1% reduction in fecal coliform for the Ulcohatchee creek. Agriculture is the main form of land use in this region and it may result in high levels of fecal indicator bacteria (FIB) in this creek. Ten farms with livestock such as cattle, goats, and horses have been identified. In another research study, cattle farming has been positively correlated with increasing FIB counts in Georgia's Oconee watershed. Increased agricultural land use in the Oconee watershed was linked to higher fecal loads from a bovine source. (Burt and Bachoon, 2013). Another likely source of FIB in the Ulcohatchee watershed is poultry, as operations housing 32,000 chickens were identified near the confluence of the creek. Other possible sources may include roadkill, illegal carcass dumping, septic systems, and wildlife such as feral pigs.

The aim of this study was to evaluate the level of fecal pollution and determine the likely source in three creeks that feed major water bodies located in the Western Piedmont region of Georgia, USA. The goals were to identify seasonal trends in FC levels and use Microbial Source tracking (MST) to pinpoint the likely sources of fecal pollution. The PCR markers used for each creek were chosen by the River Valley Region Commission (RVRC) (Georgia USA) in accordance with the most likely contaminating factors and human activity in that area. A total of 19 sampling sites (10 Longcane; 5 Ulcohatchee; 4 Mount Oak) were sampled over the course of

a year and longer in case of the Mountain Oak Creek. Information attained in this study will be beneficial in accessing public health risks from recreational activities in these creeks.

Methods

Sampling Sites

Water samples from the three watersheds were collected by the RVRC. Samples were taken monthly from December 2018 to April 2020 in the Mountain Oak creek, Oct 2018 to Sep 2019 for Long Cane, and from Jul 2018 to Feb 2019 for Ulcohatchee. Up to 200-500ml of sample water was collected in sterile polypropylene bottles at a depth of approximately 10 cm from each site. Out of 5 total sites at the Mountain Oak creek, sampling was performed at 4 targeted sites excluding site 2 (Piedmont Lake) (Fig 1). Sampling was performed at 5 sites in the Ulcohatchee creek (Fig 2) and 10 sites along the Long Cane creek (Fig 3). After collection, the samples were kept refrigerated at 2 °C to 8 °C and shipped on ice for processing to Georgia College and State University, Milledgeville.

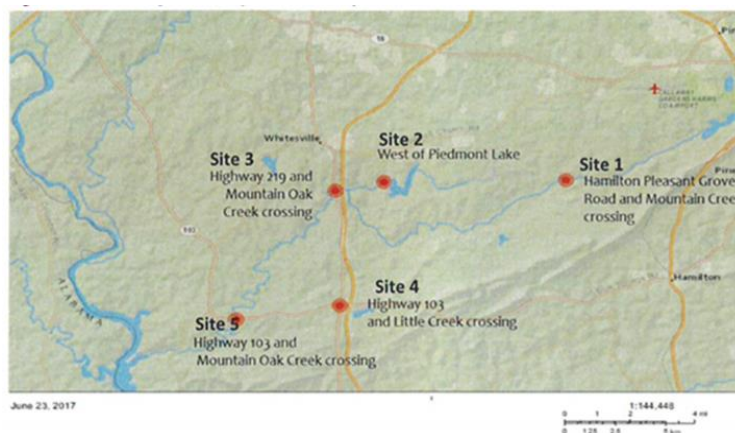


Figure 1: Mount Oak watershed water sampling sites map.

Fecal coliform enumeration and DNA extraction

Research has shown that *Escherichia coli* is a more reliable indicator for determining pathogenic bacteria in water and has since been recommended by the USEPA for management programs. However, the Georgia EPD uses fecal coliform as a standard for enumeration of fecal contamination, therefore we elected to use it in our research. To characterize the risk to human health, the EPA recommends that FC levels be less than 200 MPN/100ml in recreational waters (US EPA, 1976). Sites exceeding this value are deemed unsafe for recreational purposes. For fecal coliform enumeration, 100 ml of sample was analyzed using USEPA Standard Method 9223, Colilert TM and sealed using Quanti-Tray sealer. The trays were incubated at 44.5°C for 18 to 24 hours (USEPA, 2007). Yellow wells were considered positive for fecal coliform colonies and total counts were determined using quantification tables from the IDEXX Laboratories Inc., Westbrook, ME, USA). For DNA extraction, 100 ml of water sample was filtered through a 0.45-µm-pore nitrocellulose membrane filter (Type GS, Millipore, Billerica, MA, USA). The filters were frozen at (-20 0°C) stored until further processing. The genomic DNA was extracted using the MoBio Ultraclean Soil DNA Kit (Carlsbad, CA, USA) with modifications to the protocol (Bachoon et al., 2010; Rozier et al., 2015). The extracted DNA was quantified with a Nanodrop ND-1000 Spectrophotometer.

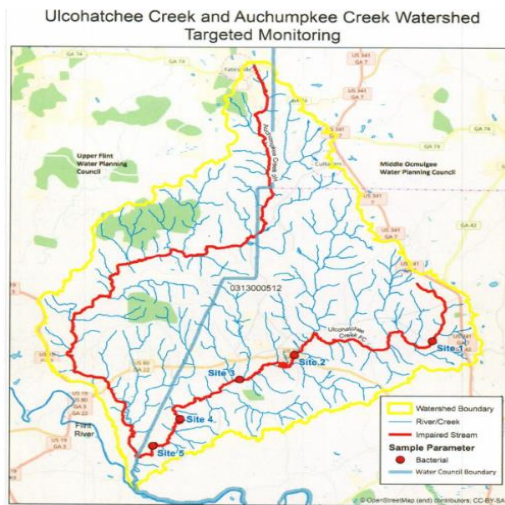


Figure 2: Ulcohatchee watershed water sampling site map.

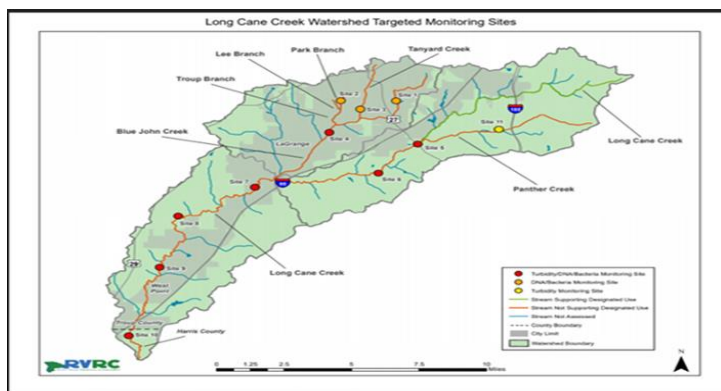


Figure 3: Long Cane watershed water sampling sites map.

Microbial Source Tracking

MST was performed bimonthly (February 2019-March 2020) for the Mountain Oak creek, once every three months (Oct 2018-May 2019) for the Long Creek, and in the same months as FC enumeration for the Ulcohatchee creek. Quantitative PCR assays for source

tracking were performed on a CFX 96 Bio RAD (Hercules, California 94547, USA) Real-Time PCR to detect human and animal specific *Bacteroides* marker genes. The primers listed in (table 1) were optimized to avoid non-specific cross reaction against not-target DNA. Annealing temperatures for each primer and probe sequences were determined from literature listed in table 1. The PCR assay for each sample was performed in duplicates with a total volume of PCR mixture of 20ul in each 200-microliter optical tube. For horse and chicken, an EvaGreen based-dye assay was used and contained a 20ul PCR mixture of 2x SsoFast EvaGreen Supermix (Bio-Rad, Hercules, CA), 25 mM of magnesium chloride: total volume 1.6 ul, and 0.1ul of 50 picomole of primers. For human, cattle, pig, canine, beaver, and deer, TaqMan assay was used, which comprised of 20ul of PCR mixture: 2 X TaqMan Probe PCR master mix (Qiagen, Valencia, CA), 2mg/ml of bovine serum albumin (BSA), 50 picomole primer and 10 picomole probe concentration. The PCR was performed under the following conditions: initial denaturation at 95°C for 10 min; 40 cycles of 95°C for 30 s, annealing temperatures varied to match the T_m of primer-pairs for each assay. For EvaGreen assays, melt curve temperatures were used to verify positive detection. For probe-based assays, comparison of C_q values to the positive control for each bacterium was used to determine presence of the specific pathogen in the sample DNA. The lower limit of detection is described in previous research and was determined by using serial dilutions of positive control DNA (Winchell et. al 2010). To evaluate for the presence of PCR inhibitors, undiluted sample DNA was compared to its ten-fold diluent and a C_t shift of <3 indicated no significant inhibition.

Table 1: Primers and probes for microbial source tracking.

Target	Primer	Sequence	Annealing Temp.(°C)	Reference
Human	HF-183-1	ATCATGAGTTCACATGTCCG	60	Haugland et al. 2010
	BtheR1	CGTAGGAGTTTGGACCGTGT		
	PROBE	6FAM-CTGAGGAGAAGGTCCCCACATTGGA-TAMRA		
Cattle	CowM2F	CGGCCAAATACTCCTGATCGT	60	Shanks et al. 2008
	CowM2R	GCTTGTTCGCTTCCTTGA		
	PROBE	FAM-TTTGCATTGAGCATCGAGGCC-TAMRA		
Chicken and other birds	CP2.9F	GTAAGACAGCAACCCCATGTA	64.5	Lu et al. 2007
	CP2.9R	ACCTATGGTTCAACACGCTTTA		
Horse	HoF597F	CCAGCCGATAAATAGTCGG	62	Dick et al. 2005
	Bac708R	CACATGTTCTCCGCTCGTA		
Beaver	Beapol-F02	AGCATTTTTCAAGCTGCTT	59	Marti et al. 2013
	Beapol-R01	ACTTAATGCCATCCCGTATTAA		
	PROBE- BEAPOL-P	HEX-CAACCTACCGTTTACTCTCGG		
Deer	Rum2-bacF	ACAGCCGCGATTGATACTGGTAA	60	Bradshaw et al. 2016
	Rum2-bacR	CAATCGGAGTTCTTCGTGAT		
	PROBE	FAM-ATGAGGTGGATGGAATTCGTCGTGT-TAMRA		
Canine	Bac Can 545F	GGA GCG CAG ACG GGT TTT	64	Kildare et al. 2007
	BacUni690 R	AAT CGG AGT TCC TCG TGA TAT CTA		
	PROBE	6FAM-ATTCGTGGTGTAGCGGTGAAATGCTTAG		
Pig	Pig-2-BacF	GCATGAATTTAGCTTGCTAAATTTGAT	60	Mieszkin et al. 2009
	Pig-2-BacR	ACCTCATACGGTATTAATCCGC		
	PROBE	TCCACGGGATAGCC		

Results

Fecal Coliform enumeration

For FC enumeration a similar pattern was observed in the three watersheds. In the Mountain Oak creek, fecal counts were highest in the summer months with the highest value of 1986.3 MPN/100ml in June 2019 at site one (Hamilton Peasant Grove Rd.) (Fig 4). This site had an overall average of 376.3 MPN/100mls throughout the entire sampling period (Dec 2018-Mar 2020). Values exceeding the USEPA standard maximum value of 200MPN/100ml were recorded on 4 sampling events in June, August, October, November of 2019. Site 2 had an overall mean of 137.0 MPN/100ml and values exceeding SSM. were recorded on two accounts in February (360.4 MPN/100ml) and October (488.4 MPN/100ml) of 2019. Site 3 had the lowest average (68.5 MPN/100ml) of the 4 sites with the highest fecal count of 209.8 MPN/100ml documented in September of 2019. Site 4 had an average of 120.7 MPN/100ml in which three months (Feb, July, Aug) of 2019 exceeding the SSM. However, these counts were marginally over the standard value and registered under 250 MPN/100ml.

In the Long Cane creek, FC levels were higher in the warmer months with the highest value of 933.33 MPN/100ml recorded in September 2019 at site 2 (Fig 5). Throughout the year-long monthly sample period, site 2 had the highest overall average of 263.9 MPN/100ml, followed by site 4 at 147.2 MPN/100 ml, and site 3 at 138.9 MPN/100ml. Fecal coliform levels at site 2 exceeded the standard on 4 different sampling events with the following highest to lowest values during these sampling dates: (933.3 MPN/100ml Sep 2019; 733.3 MPN/100ml May, 2019; 400.0 MPN/100ml July 2019; 300 MPN/100ml Oct, 2018). At site 1, the standard was exceeded only in Oct 2018 (300 MPN/100ml) and Apr 2019 (233.3 MPN/100ml).

The highest value for site 3 (300 MPN/100ml) was recorded in Oct 2018 and the standard was surpassed on two other occasions in April and May of 2019: Value (266.6 MPN/100ml). Site 4 had a maximum value of 333.3 MPN/100ml which was recorded in Feb 2019 with the second highest value of 233.3 MPN/100ml in April of the same year. The maximum value at site 5 (800.0 MPN/100ml) was recorded in April with no other sampling event exceeding the standard for that site. There were 2 values that were above the maximum standard at site 6: (300 MPN/100ml May and 233.3 MPN/100ml Sep), 1 value at site 7 (233.3 MPN/100ml Sep), and 1 value at site 8 (433.3 MPN/100ml Aug). Surprisingly, the fecal coliform standard at sites 9 and 10 was never exceeded. Interestingly, in November of 2018 and January, March, and June of 2019 the fecal counts were below this value at all 10 sites in this watershed.

The Ulcohatchee creek is part of the upper flint river watershed and was placed on the Section 303(d) list by the GA EPD in violation of the state's standard for fecal coliform. Five sites along this creek were sampled in months of July, August, October, and December of 2018 and Feb of 2019 (Table 6). The highest count of 1299.7 MPN/100ml was recorded in the month of August at site 1 (Oak grove Church road. This site had the greatest overall average (519.7 MPN/100ml) and it also exceeded the SSM standard during each sampling event. Site 2 had the second highest average 276.1 MPN/100ml with the maximum value of 410.6 MPN/100ml recorded in August. All dates except for February exceeded the state standard for fecal coliform at this site. Site 3 had had a slightly lower average of 238.7 MPN/100ml with a maximum value of 387.3 MPN/100ml in July and 2 other values exceeding the standard in August and December. Site 4 had the lowest overall average of 158.1 MPN/100ml with a maximum value of 214.3 MPN/100ml in August. The mean for site 5 was 168.3 MPN/100ml with two sites exceeding the state standard.

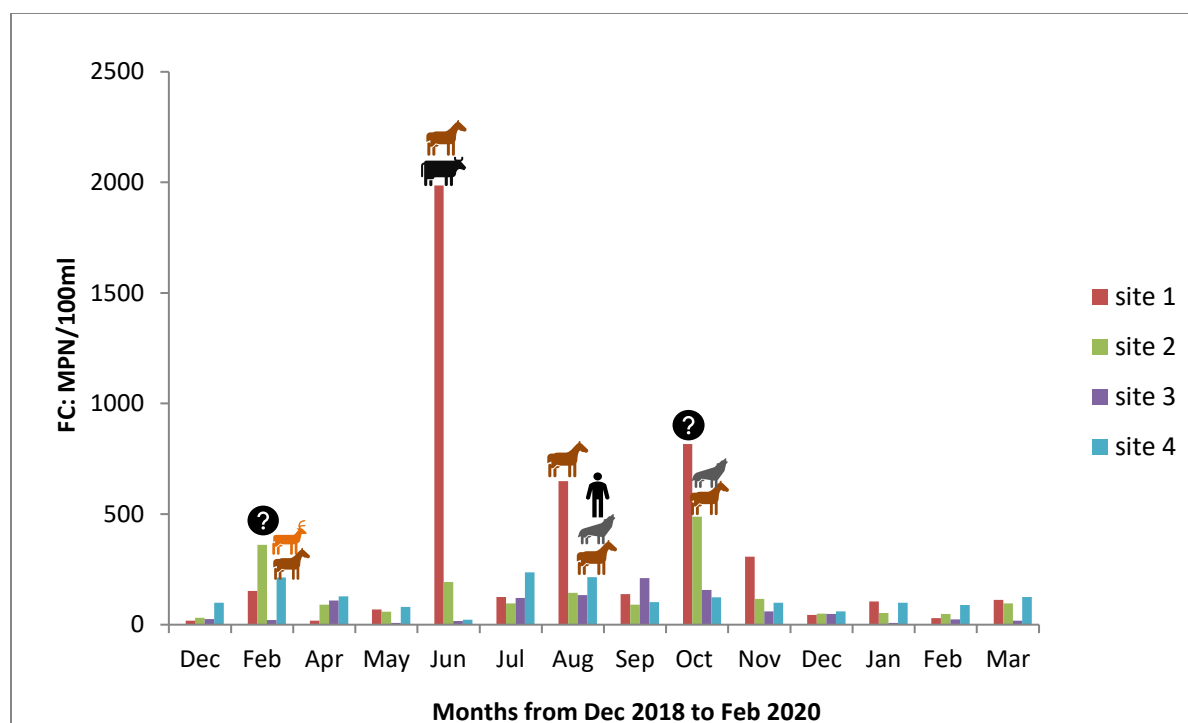


Figure 4: Fecal coliform enumeration of Mountain Oak creek. Each icon represents the source of bacteria that was determined by MST analysis. Human: ; Cattle: ; Deer: ; Horse: ; Canine: ; Unknown:

Microbial Source Tracking

The Mountain Oak creek was assessed for human, cattle, ruminant (deer), horse, and canine as potential sources of FIB. Sampling for source tracking was performed eight times bi-monthly from December 2018 until February 2020. Overall, the main contributor of FIB was horse with deer and canine being slightly less frequent, and cow and human to an even lesser extent. Source tracking results indicate that the high fecal coliform counts in June 2019 at site 1 was attributed a bovine or equine source (Figure 4). Horse was the only contributor at site 1

during August 2019 when the FC levels were 648.8 MPN/100ml. In October 2019, site 2 had high FC levels and source tracking results showed that horse and canine were potential sources. During this event, site 1 had a FC level of 816.4 MPN/100ml which is well over the SSM, yet the potential source was not identified by using the five microbial markers. Detection frequency for the different assays at the four Mountain Oak sites are shown in Table 2.

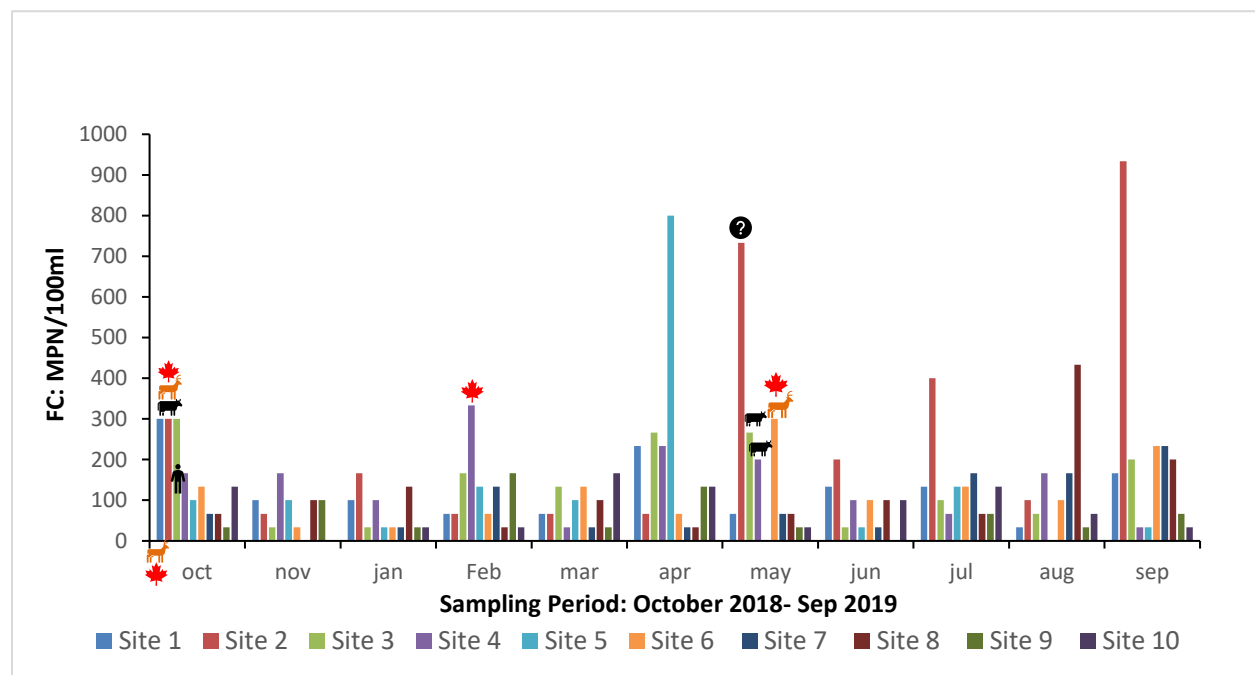


Figure 5: Fecal coliform enumeration of Long Cane creek. Each icon represents the source of bacteria that was determined by MST analysis. Human: ; Cattle: ; Deer: ; Beaver ; Unknown:

For the Long Cane creek, MST was performed during the months of October 2018, February 2019, and May 2019 to assess the source of FIB from human, cattle, ruminant, and beaver. This creek travels through the heavily populated metropolitan city of Lagrange. The main source of FIB was beaver, with ruminant and cattle contributing less and the human marker

only detected once at site three in October 2018. Site 2 in May 2019 had an FC level of 733.3 MPN/100ml. The source of FC could not be determined using the 4 microbial markers used for MST at this site. Although FC levels were high during months of April, July, August, and September of 2019, MST analysis was not performed.

The Ulcohatchee creek is part of the upper Flint and middle Ogmulgee river watersheds and travels through Crawford county. Targeted monitoring to assess for fecal coliforms and source tracking was conducted at five sites during the months of July 2018, August 2018, October 2018, December 2018, and February 2019 (Table 3). In this watershed, cattle and deer were the main sources of FIB. The Avian marker was also present at 3 different sites (1, 4, 5) during a single sampling event (July). Google map observation showed evidence of grazing cattle located near site 1 (Oak Grove Church Rd.) directly east of the Ulcohatchee creek. Since site one had the highest overall average FC counts, we conclude that FIB from cattle observed near this site were the main source. In July, the FC standard value was exceeded at sites 1, 2, and 3. Source tracking results showed that this was mainly attributed to cattle (Figure 6). In August, site 1 had a high FC value of 1299.7 MPN/100ml. Surprisingly, the potential source was not identified using the 4 MST markers at this site. Google Map observation also revealed a beef/chicken/pig producing farm situated West of Highway 80/22 (Site 2) of the Ulcohatchee creek. However, the pig marker was not detected at any of the sites during the sampling period (Table 3) (Figure 6).

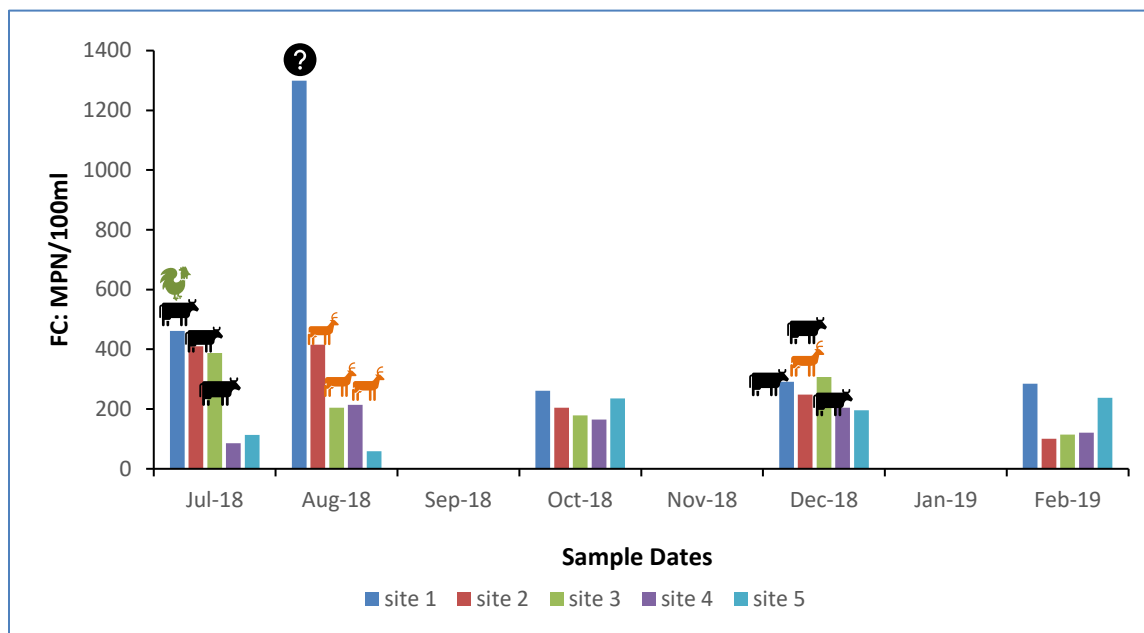


Figure 6: Fecal coliform enumeration of the Ulcohatchee creek. Each icon represents the likely source of bacteria that was determined by MST analysis. Cattle: ; Deer: ; Pig: ; Chicken: ; Unknown:

Discussion

Fecal pollution in aquatic environments is determined by the presence of FIB. The Clean Water Act of 1972 was established to regulate discharge of pollutants into water. Section 303 (c) of the act addresses Total Maximum Daily Load (TMDL) which is defined as the maximum pollutant load a watershed can receive before it is deemed impaired. Fecal coliform bacteria have been the preferred and primary indicator bacteria for Federal and State regulatory agencies until 1986 when *E.coli* became the preferred FIB for testing water pollution. However, the Georgia Environmental Protective Division (GA EPD) still recommends the use of FC over *E. coli* for

testing recreational water. In this study, many sites of the three sampled watersheds exceed the FC Single Sample Maximum standard value of 200 MPN/100ml on multiple occasions. In the past, The GA EPD has determined many of the Chattahoochee and Flint basin stream segments to have elevated levels of FC (GA EPD, 2014).

Table 2: Microbial source tracking and detection frequency of each MST marker from eight sampling events between December 2018-March 2020 of the Mount Oak creek.

Site	Human	Cattle	Deer	Horse	Canine
Hamilton Peasant Grove Rd.	-	+1	-	+5	+1
Highway 219	-	+1	+3	+3	+4
Highway 103 Little Creek	-	+1	+1	+1	-
Highway 103 Mount Oak	+1	+1	+3	+6	+4

Research has also shown that the Chattahoochee river itself to have elevated levels of FC. Findings suggest a lower FC level upstream of the river in comparison to the downstream segments, which are fed by tributaries and pass through metropolitan Atlanta (Gregory and Frick, 2000). A study by Schoonover and Lockaby (2006), assessed the relationship between land cover and water quality in 18 mixed land use watersheds in the southern Piedmont region of west Georgia. Increasing land cover was attributed to higher levels of fecal coliform bacteria. Another study found high FC levels ranging from (1100 CFU mL⁻¹ to 375 CFU mL⁻¹) in the Savannah river and its adjacent lakes between July 2013 and February 2014 (Williams et al. 2016). They correlated it with high turbidity, which was not assessed in our study. High levels of

FC bacteria have been detected in other surface waters such as the Oconee river in Georgia. The Oconee watershed, like the Flint and Chattahoochee, is in the Piedmont region and it is subjected to stress from urbanization and agriculture. In this watershed, confined animal production and dairy production plants can be a source of conflict due to proximity with municipal water supplies and recreational areas. Increased nutrient loading from field applications of manure from animal operations can be an environmental hazard. Research shows that the Upper Oconee to has a high amount of FIB. Fisher et al. (2000) recorded high nutrient and FC concentrations in both the river portions of the watershed as well as Lake Oconee itself. Unlike our research, these studies did not evaluate water quality in relation to the sources of the bacteria.

Table 3: Microbial source tracking and detection frequency of MST marker from five sampling events between July 2018-February 2019 of the Ulcohatchee creek.

Site	Chicken	Pig	Ruminant	Cattle
Oak Grove Church Rd	+1	-	-	+2
Highway 80/22	-	-	+2	+2
Julia Jordan rd	-	-	+2	+2
White Taker Rd	+1	-	+3	-
Reeve Road	+1	-	-	+1

In this research, FC enumeration along with detection of human and animal *Bacteroides* in three recreational watersheds was performed over a prolonged period. Results indicated a possible relationship between FC levels and the presence of *Bacteroides*. Assessment of cq

values in positive samples for each *Bacteroides* markers were used to determine DNA quantification. Smaller cq values are attributed to early amplification and larger abundance of the host-specific 16s rRNA gene. As determined by serial dilution, a cq shift ≤ 3 is equivalent to a 10-fold increase in DNA concentration. In this research, values less than 35 were considered positive for each marker. Although a significant relationship between FC and *Bacteroides* could not be determined, a cq value (15.05) for the CowM2 marker was detected at site 1 of Mountain Oak creek in June. The FC levels at this site and date were 1986.3 MPN/100mls. A similar pattern occurred during October 2019 in the Mountain Oak creek. During this event, the canine marker was detected at site 2 (cq: 15.98); FC level (488 MPN/100ml). Schriewer et al. (2015) demonstrated a positive correlation between FC and animal *Bacteroides* markers in community water sources and stored drinking water in rural India. However, no correlation between the human marker and FC was determined in that study.

FC bacteria and *Bacteroides* marker concentrations may vary in rivers and streams in response to changing environmental conditions as well as different land use practices (Schoonover et al. 2005) (McKee, et al. 2020). The MST markers in this research used in testing of each creek were considered the most probable contaminating factors in those regions as selected by the River Valley Regional Commission in Columbus, Georgia. McKee et al. (2020) assessed differences in concentration of human, dog, and ruminant markers in the Chattahoochee River National Recreational Area in metropolitan region of Georgia. Levels of the tested markers, excluding ruminant, were higher in wet (high precipitation) conditions. High stormflow conditions and increasing impervious surface also contribute to greater fecal coliform levels in rivers. Urban and rural areas of the Georgia's western Piedmont have an increase in FC levels in comparison undeveloped or forested regions (Schoonover et al. 2005) (Gregory and Frick 2000).

Our study assessed FC levels in both rural (Mountain Oak/ Ulcohatchee) and urban (Long Cane) streams and the results correspond with those of Schoonover and Gregory. Monthly average rainfall assessment of areas surrounding the three watersheds in this study shows a lack of significant correlation between precipitation and FC enumeration. However daily rainfall assessment was not performed before each sampling event. Therefore, we cannot assume that the high levels of FC in these watersheds were not attributed to increased runoff resulting from storm events. Rather than rainfall, warmer weather was a better indicator of FC and *Bacteroides* persistence in this research.

It is very important to determine the source of fecal pollution in an environment to take the appropriate actions to reduce or stop the flow of FIB in a system. Other research studies have examined sources of bacteria using Microbial Source Tracking techniques. Heaney et al. (2015) demonstrated the adverse effect of large, concentrated swine feeding operations in North Carolina using FIB enumeration and MST. Fecal coliform levels at tested sites often exceeded the guideline set by the USEPA. The swine markers Pig-1-Bac and Pig-2-Bac showed 100% specificity and were detected in 17% and 14% of the tested samples (Heaney et al. 2015). This study examined FIB loads from a swine source in proximity to nearby streams in the Coastal region of North Carolina. This region has a large amount of swine production in comparison to Georgia's western Piedmont. Therefore, high FIB from a swine source in Georgia would most likely result from wild hogs rather than domestic pigs.

Quantitative PCR assays for testing for a variety of different sources were used in this research. *Bacteroides* species are characterized as obligate anerobic species which represent one-third of the human fecal bacteria (Holdman et al. 1976). The Hf183 marker has been widely used and accepted as a highly specific Bacteroidale marker for targeting human fecal contamination

(Rozier et al. 2015; Krentz et al. 2013). We did not detect a significant amount of this marker in the three tested watersheds. We attribute this to the effective management and proper disposal of human wastes in urban areas in the Long Cane creek and a lack of human influence in forested and rural regions of the Mountain Oak creek. Previous research has shown the CowM2 *Bacteroidale* marker to have varying degrees of specificity and sensitivity for determining cattle specific *Bacteroides* (Harwood et al. 2014, Shanks et al., 2008). Despite this, it is widely accepted and used in various MST studies and has been reported to perform better than the CowM3 marker (Raith et al. 2013). Surprisingly, bacteria from cattle were present at a higher degree at the Lone Cane and Ulcohatchee creeks in comparison to the Mountain Oak creek, even though the latter comprises 86% of land occupied by agriculture. The cattle marker was detected in Mount Oak creek on one account at site 1 which had FC level of 1986.3 MPN/100ml. Research indicates that bovine fecal pollution persists in areas near streams adjacent to cattle grazing pastures and agricultural land (Bachoon et al. 2009) (Wilkes et al. 2013). The cattle marker was detected at this site during the same two months as site 1 (July and December 2018). This indicates that the presence of the CowM2 marker at these sites might be linked to the same source.

Table 4: Microbial source tracking and detection frequency of each MST marker from four sampling events between October 2018, Feb 2019, and May 2019 of the Long Cane creek.

Site	human	Rum	cattle	Beaver
1	-	+1	-	+2
2	-	+1	+1	+1
3	+1	-	+1	-
4	-	-	+1	+2
5	-	+2	+2	+2
6	-	+3	+1	+3
7	-	+2	+1	+2
8	-	+1	+1	+2
9	-	-	-	+3
10	-	-	+1	+2

At site 1 of the Mountain Oak creek, the average FC levels were highest throughout the sampling period. Source tracking results using the five microbial markers for human, cattle, deer, horse, and canine showed that horse was the major contributor at this site. We attributed this to the large number of rural houses harboring these domestic animals in the agricultural areas surrounding the creek. Canine microbial tracker was also observed a total of 6 times in this creek during the 8 sampling events. Source tracking showed that canines were the main contributor to high FC levels at site 2 (488.4 MPN/100ml) in October 2019. At the other 5 sites where this marker was detected, the FC levels were not significantly high. Through google map observation, we identified nearby houses adjacent to the stream sites where this marker was

prominent. Family pets instead of wild canines may have contributed to the higher counts here but we cannot disprove coyotes as a possible source.

The Rum2Bac marker was used for identifying FIB from deer in the three creeks tested. Research has shown this MST marker to be effective both in sensitivity and specificity in determining presence of fecal bacteria from ruminants such as cattle, deer, and sheep (Raith et al. 2013). In some instances, both the CowM2 and Rum2Bac markers were detected at a single site on a sampling event (i.e. Long Cane: sites 5, 6, 7; Ulcohatchee sites: 2, 3). Therefore, the conclusion was drawn that deer may not have been a major contributor to FIB at these sites. Source tracking results show that deer were not a major source of FC in the Mountain Oak creek. Excluding 1 event, (site 4: February 2019) the FC counts were below the SSM standard at all other sites where the Rum2Bac marker was detected. CybrGreen assays were used for source tracking of chicken and other birds with the *Bacteroides* CP2-9 marker in the Ulcohatchee creek. Lu et al. (2007) demonstrated the effectiveness of this marker in detecting chicken fecal contamination in rivers of Georgia, Delaware, and Ohio. In our research, we detected chicken during the month of July at three sites in the Ulcohatchee creek. However, chicken was not a major FIB source in this creek. The pig-specific Bacteroidale marker Pig-2-Bac was used to detect a porcine source in the Ulcohatchee creek. Previous research has shown this marker to have a high specificity to pigs (Gourmelona et al. 2010) (Mieszkin et al. (2009, 2010)) and (Marti et al. 2010). We did not detect pig in any of the five sampling events even though there is a swine producer near site 2 of this creek. Although Georgia is not a state characterized by a high swine production, studies have provided evidence of wild hog activity in some counties. Lama et al. (2018) demonstrated the impact of wild boars by detecting pathogenic *E. coli* and *Brucella suis* strains in 87 fecal samples that were collected across 16 Georgia counties.

In the Long Cane creek, beaver was the most significant source of FIB as it was detected at every site at least once in the 5 source tracking events. From the late 1930s through 1950s, state wildlife agencies have helped reestablish beaver population throughout the United States. Beavers are concentrated primarily in the Piedmont and Inner Coastal Plain but also distributed in smaller numbers in the outer Coastal Plain, Blue Ridge, Ridge and Valley, and Appalachian Plateau (Butler, 1991). This reintroduction into Georgia and much of the Southeast has brought many positive impacts to the wetland habitats. These impacts include reduced erosion and sediment transfer by stream damming and reducing the chances of flooding. However, an increased population around urban areas such as roadside ditches near streams may have caused high FIB transmission into the water. Marti et al. (2013) demonstrated high sensitivity and selectivity for the Beapol marker thus we ruled out the likelihood of cross-reactions with other assays.

Conclusion

Fecal pollution in recreational waters is a threat to public health in the United States. In this study, high levels of fecal coliform bacteria were detected in each of the three studied Piedmont watersheds in Georgia. The FC levels in the Mountain Oak creek were highest in the warmer months especially in June, August, and November of 2019. Source tracking results indicated the horse, ruminant, and canine markers were the likely source of bacteria in this creek. High levels of FC were detected throughout the sampling period in the Long Cane creek. The levels were particularly high in the months of April, May, and September of 2019. Despite limited source tracking data, results showed the beaver marker had the highest frequency of detection. Cattle and ruminant markers were also present throughout the sampling period in this watershed. The human marker was only detected once in both the Long Cane and Mountain Oak

creeks. Therefore, fecal contamination in these two watersheds cannot be linked to a human source. The Ulcohatchee creek followed a similar seasonal pattern in FC levels. The levels in this watershed were highest in the months of July and August. Source tracking results showed that the main contributors were cattle and deer. High fecal coliform levels in these three major watersheds call for reduction through mitigation strategies. High level of bacteria from both ruminant and equine sources were present in these watersheds. TMDL programs should focus on mitigating bacterial loads coming from horse or cattle farms near these creeks. Further research should concentrate on the enumeration of FC and identification of both human and animal sources of pollution by utilizing MST in other Georgia watersheds as well.

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